# Transcription factors ETS2 and MESP1 transdifferentiate human dermal fibroblasts into cardiac progenitors

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**Unique insights for the reprograming of cell lineages have come from embryonic development in the ascidian *Ciona*, which is dependent upon the transcription factors *Ci*-ets1/2 and *Ci*-mesp to generate cardiac progenitors. We tested the idea that mammalian v-ets erythroblastosis virus E26 oncogene homolog 2 (ETS2) and mesoderm posterior (MESP) homolog may be used to convert human dermal fibroblasts into cardiac progenitors. Here we show that murine ETS2 has a critical role in directing cardiac progenitors during cardiopoiesis in embryonic stem cells. We then use lentivirus-mediated forced expression of human ETS2 to convert normal human dermal fibroblasts into replicative cells expressing the cardiac mesoderm marker KDR$^+$. However, although neither ETS2 nor the purported cardiac master regulator MESP1 can by themselves generate cardiac progenitors de novo from fibroblasts, forced coexpression of ETS2 and MESP1 or cell treatment with purified proteins reprograms fibroblasts into cardiac progenitors, as shown by the de novo appearance of core cardiac transcription factors, $Ca^{2+}$ transients, and sarcomeres. Our data indicate that ETS2 and MESP1 play important roles in a genetic network that governs cardiopoiesis.**

cardiogenesis | fibroblast reprograming | protein transduction | kinetic imaging

The elucidation of key developmental regulatory genes has provided unique insights into reprogramming of somatic cells into cells of other lineages (1–4). For example, analysis of the ascidian *Ciona intestinalis* showed that cardiac founder cells express transcription factors *Ci*-mesp (mesoderm posterior) and *Ci*-ets1/2 (5–8). *Ci*-Mesp regulates a cardiac progenitor regulatory network, including the core factors GATA, NKX, and HAND (6). Furthermore, *Ci-mesp* knockdown embryos do not develop heart primordia (9, 10), and targeted inhibition of *Ci-ets1/2* activity blocks heart specification and the expansion of the heart field (8). *Mesp1* and its closely related gene *Mesp2* also direct the appearance of cardiac progenitors in mouse ES cells (9). However, *Mesp1* alone is ineffective in directly converting cardiac fibroblasts into cardiac myocytes (1), and therefore cannot be considered a single "key regulator" (11). Therefore, we asked whether mammalian *Mesp1* also requires *Ets2* to initiate the differentiation of cardiogenic progenitors in embryonic stem cells and potentially transdifferentiation of naive fibroblasts.

ETS2 is part of the E26 transformation-specific family of transcription factors, consisting of 27 genes in humans (12). ETS factors are transacting phosphoproteins with key roles in cell migration, proliferation, differentiation, and oncogenic transformation (13–15). ETS2 is required for the earliest and most fundamental events of murine embryonic anterior-posterior patterning, primitive streak, and mesoderm initiation from the epiblast (16–18), but its role in cardiac differentiation had not been examined. We show here that ETS2 also plays multifaceted and essential role in cardiogenesis and in combination with MESP1 reprograms human dermal fibroblasts into cardiac progenitors.

## Results

**ETS2 Is a Critical Cardiopoiesis Factor.** We compared the expression profiles of mammalian cardiomyogenic genes in wild-type E14 and *Ets2*$^{-/-}$ mouse ES cells. T-brachyury, a core T-box factor required for initiating the appearance of cardiac mesoderm was not affected by the loss of *Ets2* (Fig. 1*A*). However, *Mesp1* and *Mesp2*, the earliest markers of cardiac specification, were inhibited in *Ets2*$^{-/-}$ cells (Fig. 1*A*). *Nkx2.5*, *Mef2c*, and *Tbx5* transcription factors, and *Ryr2* and *α-MHC*, excitation and contraction genes, were also inhibited in *Ets2*$^{-/-}$ cells (Fig. 1 *A* and *B*). No rhythmic beating was initiated in *Ets2*$^{-/-}$ embryonic bodies (EBs), nor was α-actinin staining observed in *Ets2*$^{-/-}$ EBs (Fig. 1 *C* and *D*). Microarray analysis revealed 1,205 down-regulated genes (>twofold) and 1,021 up-regulated genes (>twofold) in *Ets2*$^{-/-}$ vs. E14 EBs. Gene Ontology (GO) analyses showed enriched GO terms among the down-regulated genes (Table S1) closely associated with cardiovascular system development and blood vessel morphogenesis. The heat map in Fig. 1*B* shows the reduced expression of cardiac transcription factors *Csrp3*, *Nkx2.5*, *Isl1*, *Tbx5*, *Smyd1*, and *Zic3*, and contractile proteins *Actc1*, *Myl2*, *Myl4*, and *Tnnt2* in *Ets2*$^{-/-}$ EBs. Furthermore, analysis for blood, gut, and neural derived cells revealed little changes in gene expression (Fig. S1). These data support the idea that ETS2 is essential for the development of cardiovascular system and raised the possibility that human ETS2 may drive cardiovascular gene expression in naive normal human dermal fibroblasts (NHDFs).

**ETS2 Reprogrammed Fibroblasts into Kinase Insert Domain Receptor-Positive Cells.** Constitutive ETS2 overexpression in NHDFs (Fig. 2 *A* and *B*) gave rise to highly replicative small rounded cells within 1 wk after lentiviral infection, whereas mock infections with the empty vector maintained slower growing fibroblasts (Fig. 2*C*). ETS2 induced the appearance of cell types with some of the characteristics of endothelial and cardiac progenitors that express surface markers CD31/platelet endothelial cell adhesion molecule 1 (PECAM1) (19) and CD309/kinase insert domain receptor (KDR) (20) (Fig. 2*D*). Furthermore, ETS2 was able to enrich the RNA transcripts of core components in the Activin/Nodal

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Data deposition: The sequences reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE29710).

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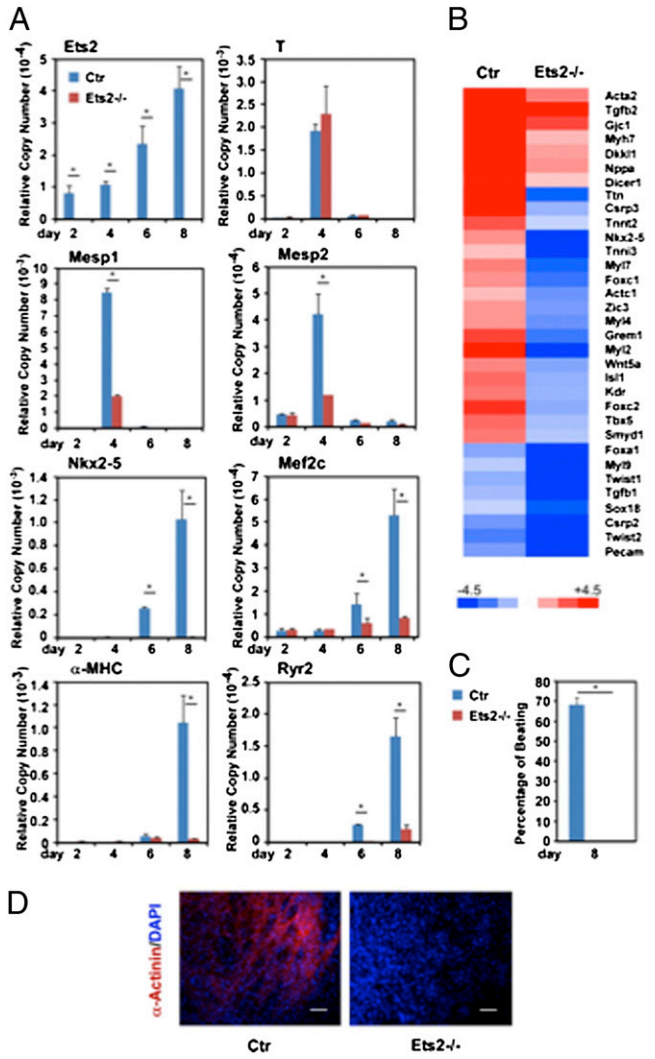


**Fig. 1.** ETS2 is essential for cardiopoesis in mouse ES cells. (*A*) qRT-PCR revealed *Ets2* expression in differentiating E14 murine postembryoid bodies, and absence of expression in *Ets2*$^{-/-}$ cells. RNA was collected at indicated time-points and analyzed by qRT-PCR using primer sets from Table S2. Expression of cardiac mesoderm markers *Mesp1*, *Mesp2*, cardiac transcription factors *Nkx2.5*, *Mef2c*, and cardiac structural genes *α-MHC*, *Ryr2* was impaired in *Ets2*$^{-/-}$ cells. *$P < 0.05$, $n \geq 3$, Student *t* test. (*B*) Heat map of representative gene expression from the whole genome microarray from day 10 postplated EBs. RNA samples were hybridized against Affymetrix array 430a2 chips. Microarray raw data analysis was done with dCHIP software (http://biosun1.harvard.edu/complab/dchip/). (*C*) Spontaneous beating was absent in *Ets2*$^{-/-}$ EBs. *$P < 0.05$, $n \geq 3$, Student *t* test. (*D*) Lack of α-actinin immunostaining in day 10 *Ets2*$^{-/-}$ EBs. (Scale bar, 50 μm.)

program (*ACVR2B*, *TDGF1*, *CITED2*, and *LEFTY1*) (Fig. 2*E* and Fig. S2). The Activin/Nodal pathway is obligatory for the appearance of cardiac progenitors and in its absence cell fate is shifted toward a neural program (21, 22). Expression of the core cardiac transcription factors, including *NKX2.5*, *MESP1*, *MEF2C*, *GATA4*, and *ISL1* was also stimulated by ETS2, consistent with the study showing that ETS factors regulate *GATA4* (23). Furthermore, overexpression of MESP1 by lentiviral infection of NHDFs did not radically change cell shape (Fig. 2*C*) but activated the appearance of KDR and a few cardiac transcription factors (Fig. 2*E* and Fig. S3). Like ETS2, MESP1 blocked the appearance of bone morphogenetic protein 2 (*BMP2*), *BMP4*, and *PDGFRα* (Fig. 2*E*), which are associated with the induction of the cardiac progenitor program in ES cells (24, 25). These KDR$^+$ and PECAM1$^+$ cells did not induce terminal cardiogenesis.

**ETS2/Mesp1-Induced Conversion of NHDFs into Cardiac Progenitors.** As shown in the experimental protocol (Fig. 3*A*), we tested whether both ETS2 and MESP1 have to be coexpressed to reprogram NHDFs and whether Activin A and BMP2 maximize the induction of cardiac gene expression, as previously shown for KDR$^+$ ES cells (20). To follow *NKX2.5* activation in cells, we used lentiviral NKX2.5-tdTomato reporters, which showed strong fluorescence activity in beating myocytes derived from mouse ES cell EBs (Fig. S4). We achieved tight control over ETS2 and MESP1 expression, by using a doxycycline (Dox)-regulated system (Fig. S5). To optimize test conditions, ETS2 and MESP1 were simultaneously coexpressed for 2, 4, and 6 d followed by 2-d stimulation with Activin A and BMP2 and further cultured in α-MEM media. We observed green fluorescence for viral vector-driven GFP and red fluorescence for activated NKX2.5-driven tdTomato reporter activity, which showed conversion of dozens-to-hundreds of NHDFs at multiple locations (colonies) by 2–4 d of Dox-treatment (Fig. 3*B*). Whereas the 6-d coexpression gave rise to three to five tdTomato$^+$ colonies per plate, the best conversion rate (approximately 30 tdTomato$^+$ colonies per plate) was observed when ETS2 and MESP1 were coinduced for 4 d.

Under optimized conditions (Fig. 3*C*) we observed virtually no induction of core cardiac transcription factors, signaling factors, and contractile proteins in uninfected or empty Dox-inducible vector-infected NHDFs. The addition of Activin A and BMP2 for 2 d did not stimulate expression of cardiac-specified genes in fibroblasts (Fig. 3*C*). Individual Dox-induction of ETS2 or MESP1 caused some induction of *NKX2.5*, *GATA4*, and *TBX20* but failed to up-regulate *TNNT2* and Ca-α-actin, even after Activin A and BMP2 treatments (Fig. 3*C*). The strongest induction of *MEF2C* and *HAND2*, *TNNT2*, and Ca-α-actin occurred with the coinduction of ETS2 and MESP1, followed by Activin A and BMP2. Furthermore, coexpression of ETS2 and MESP1 induced BMP2 expression over 20-fold but separately they had no effect (Fig. 3*C*). In addition, a heat map of RNA from the latter optimized 8-d culture analyzed by quantitative RT-PCR (qRT-PCR) showed significant up-regulation of core cardiac factors, as well as contractile proteins (Fig. 3*D* and Fig. S6). The addition of Activin A or BMP2 for 2 d for the most part stimulated expression of cardiac-specified genes in fibroblasts.

**TAT-ETS2 and TAT-MESP1 Proteins Reprogram NHDFs into Immature Cardiomyocytes.** To avoid the known concerns about deleterious rearrangements of the host chromosomes during viral integration (26, 27), we asked whether protein transduction technology could directly reprogram NHDFs (Fig. 4*A*). ETS2 and MESP1, fused to a short basic fragment of transactivator of transcription (TAT) protein as a cell-penetrating peptide (28), were expressed in *Escherichia coli*, purified by the 6xHis affinity chromatography (Fig. 4*B*) (TAT-ETS2, 60 KDa; TAT-MESP1, 38 KDa), and applied in serum-free culture medium at 50-nM concentrations for four daily cell treatments. Immunohistochemical reaction with the HA epitope revealed accumulation of TAT-ETS2 and TAT-MESP1 in cellular nuclei (Fig. 4*C*). Colonies of cellular aggregates appeared only after the combined ETS2 and MESP1 protein treatment and up-regulated cardiogenic myosin light-chain 2v (MLC2v) reporter activity (Fig. S7*B*) and NKX2.5-tdTomato$^+$ cells were detected within 8 d (Fig. 4*D*). Similarly, in Western blots with specific antibodies we observed substantial induction of α-striated actin, TNNT2, TNNI3, and to a lesser extent MHC expression in FACS-sorted NKX2.5$^+$ cells (Fig. 4*E*). FACS analysis revealed about 9% conversion rate of the NHDFs into tdTomato$^+$ cells (Fig. 4*F*). Approximately 91% of these cells showed KDR/PECAM1 cell surface markers representing cardiac mesodermal cells according to Kattman et al. (29). None of the cells displayed only PECAM1 marker, making it unlikely that NKX2.5 was expressed in endothelial cells. To determine whether the ETS2/MESP1 cell treatment could generate functional cardiac myocytes, NHDFs were first preinfected with α-MHC-Puro selection lentivector (30) followed by the optimized conditions for TAT-protein transduction. After 8 d, cells were treated with 10 μg/mL puromycin to select converted cells. Maturation of selected cells for 2 wk revealed the appearance of striated thin filament staining detected with a striated α-actin antibody (Fig. 4*G*).

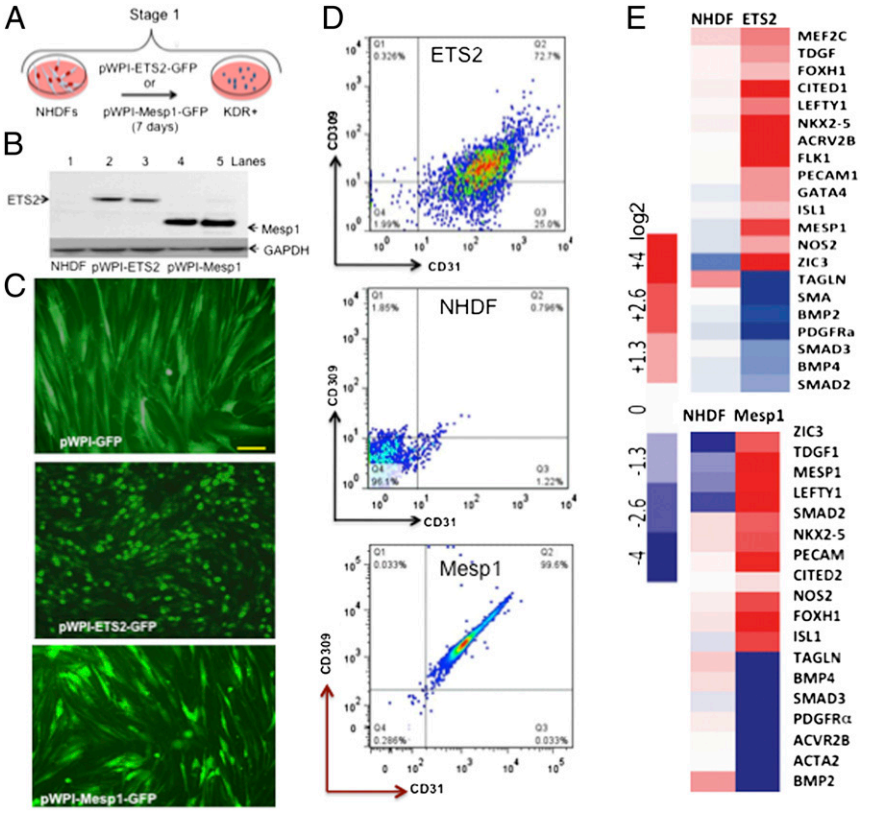


**Fig. 2.** ETS2 and MESP1 induced low passage NHDFs into KDR1$^+$ cells. (*A*) Experimental protocol. (*B*) Western blot to verify constitutive expression of ETS2-HA and MESP1-HA proteins detected with HA-antibodies. (*C*) ETS2 (pWPI-ETS2-GFP), Mesp1 (pWPI-Mesp1-GFP), and control (pWPI-GFP) lentiviruses were infected into StemPro-cultured NHDF and allowed to express for 1 wk. Cell morphological changes were observed by the GFP fluorescent images. (Scale bar, 50 μm.) (*D*) FACS analysis revealed enrichment of cardiovascular surface markers CD31 (PECAM1) and CD309 (KDR) in ETS2-infected cells (*Top*) and Mesp1-infected cells (*Bottom*) in comparison with empty vector-infected cells (*Middle*). (*E*) Heat maps of qRT-PCR from mRNA isolated from infected cells after 7 d in culture.

To follow the maturation of reprogrammed cells, they were also analyzed with the multichannel kinetic imaging cytometer (KIC) capable of monitoring many individual spontaneously contracting cells for fluorescence signals with Fluo-4 as an intracellular $Ca^{2+}$ indicator (31). First, under optimized Dox-induced conditions, as shown in Fig. 3*A*, ETS2 and MESP1 individually were ineffective

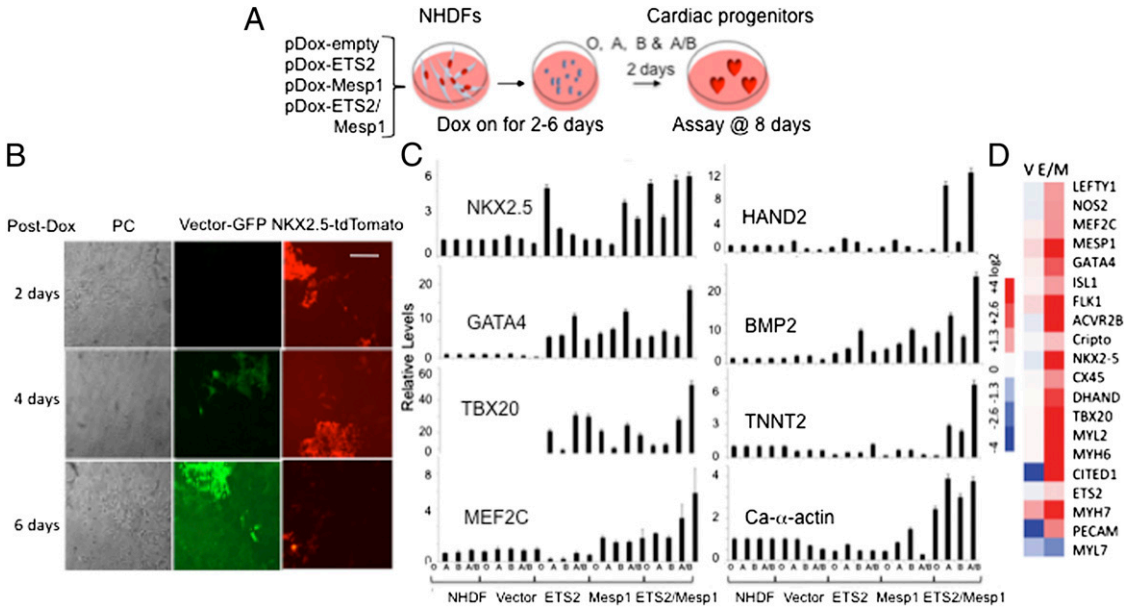


**Fig. 3.** Dox-induced viral ETS2 and MESP1 expression activated the cardiac program in NHDFs. (*A*). Experimental protocol. (*B*) To optimize test conditions, NHDFs were preinfected with NKX2.5-tdTomato reporter and rtTA2 vectors and then infected with pDox-ETS2-GFP and pDox-Mesp1-GFP and cultured in α-MEM with supplements. Green fluorescence is a marker for Dox-induced viral expression and red fluorescence shows NKX2.5-tdTomato reporter activation. (Scale bar, 250 μm.) (*C*) Individual components and expression vectors were tested under optimized 4-d Dox induction, in the absence (O) and presence of Activin A (A) and BMP2 (B). RNA was collected at 8 d after beginning of Dox treatment and analyzed by qRT-PCR using primer sets from Table S3. (*D*) A heat map of RNA from the latter optimized 8-d culture analyzed by qRT-PCR shows significant up-regulation of core cardiac factors in cells infected with an empty vector virus (V) or with a combination of viruses carrying *ETS2* and *Mesp1* genes (E/M).

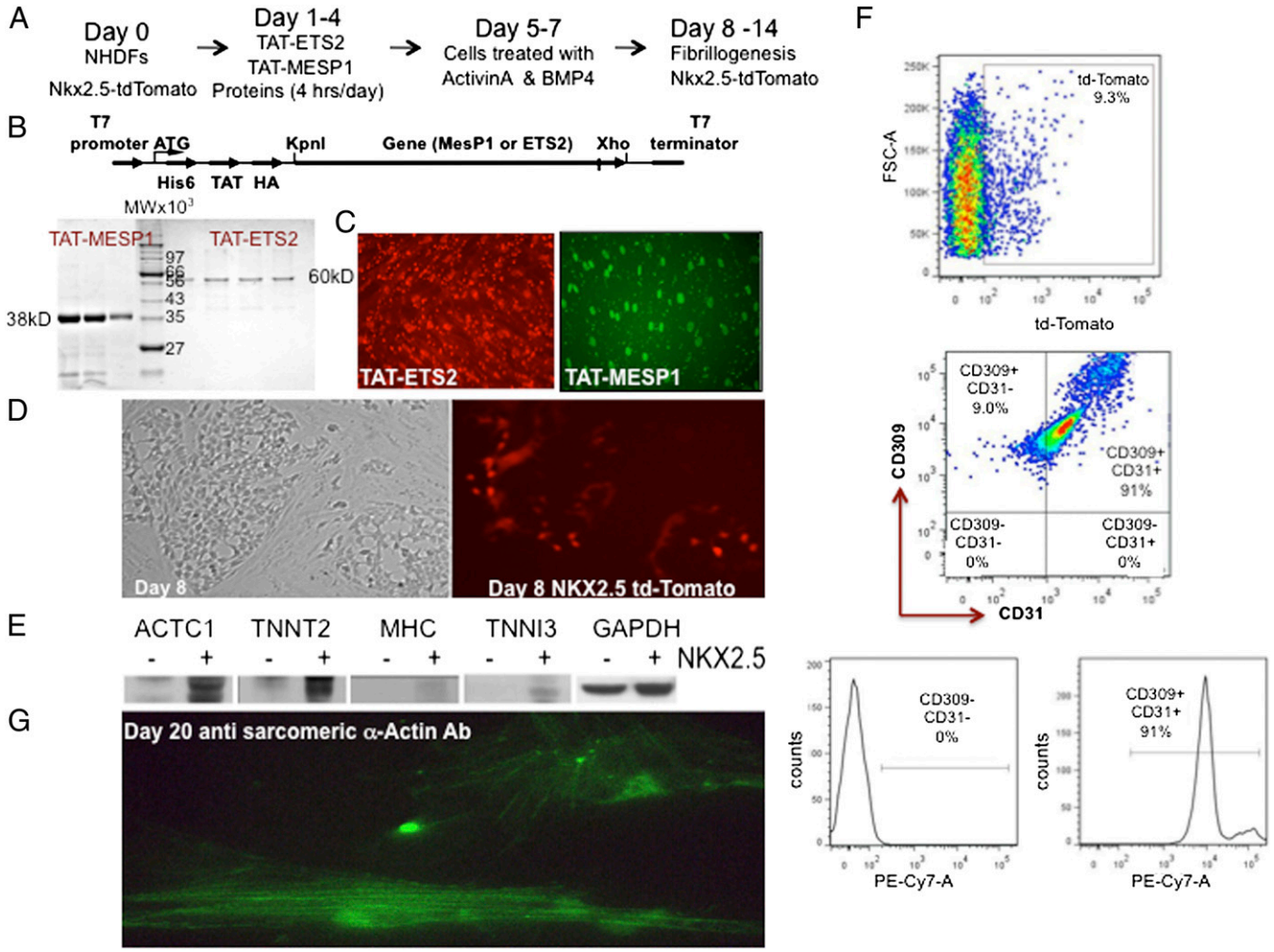


**Fig. 4.** Protein transduction with ETS2 and MESP1 factors reprogrammed fibroblasts into cardiac myocytes. (*A*) A reprogramming protocol using purified TAT-ETS2 and TAT-MESP1 to transduce NHDFs. (*B*) Schematics of the bacterial expression vector shows the TAT-tag used for the protein uptake into NHDFs, the 6xHis-tag for affinity purification, and the HA-tag for immunofluoresence. TAT-protein fractions purified by 6xHis affinity chromatography were analyzed by SDS/PAGE (TAT-ETS2, 60 KDa; TAT-MESP1, 38 KDa). (*C*) Immunohistochemical reaction against the HA epitope reveals either TAT-ETS2 or TAT-MESP1 accumulation in cellular nuclei 16 h after treatment at 50-nM concentrations. Magnification, 20×. (*D*) Within 8 d after the beginning of combined ETS2 and MESP1 protein treatment, NKX2.5-tdTomato$^+$ cells appeared in colonies of cellular aggregates. Magnification, 10×. (*E*) Induction of α-striated actin, cTnT, and TnI expression, and to a lesser extent MHC expression from FACS-sorted NKX2.5$^+$ cells assayed by Western blots with specific antibodies. (*F*) FACS analysis revealed a conversion of about 9% of the NHDFs that were tdTomato$^+$. These cells showed KDR (CD309)/PECAM1(CD31) cell-surface markers of cardiac mesodermal cells. (*G*) NHDFs were preinfected with α-MHC-Puro selection lentivectors following by TAT-protein reprogramming conditions. After 8 d, cells were treated with 10 μg/mL puromycin to select converted cells. Maturation of selected cells for 2 wk revealed the appearance of striated thin filaments with striated α-actin antibody staining. Magnification, 60×.

even with growth factors, but in combination they induced significant beating activities (Fig. 5*A*). Next, we used the optimized TAT-protein transduction conditions, as shown in Fig. 4*A*, and the viral α-MHC-Cherry reporter (30), a marker of more advanced cardiac development than NKX2.5, was preinfected into NHDFs. α-MHC-Cherry FACS-enriched cells comprised 13.7% of the total population (Fig. 5*B*). Fig. 5*C* shows the software-assisted image segmentation into individual cells for single-cell analysis of $Ca^{2+}$ transients. Representative $Ca^{2+}$ recordings as changes in Fluo-4 intensity for cells identified in Fig. 5*D* are shown in Fig. 5*E* and in Movies S1, S2, and S3. Characterization of KIC-analyzed cells also revealed the total amount of α-MHC-Cherry$^+$ cells analyzed (5,635), the total amount of cells in which $Ca^{2+}$ transients were detected (984), amount of cells with a single $Ca^{2+}$ peak (857), and the total amount of cells with two or more $Ca^{2+}$ peaks (127) (Fig. 5*F*). Thus, 17% of the α-MHC-Cherry$^+$ cells were contractile amounting to a myocyte conversion rate of 2.3% of starting NHDFs. Finally, many of the α-MHC-Cherry$^+$ cells stained with α-striated actin antibody displayed periodic staining (Fig. 5*G*) highly reminiscent of embryonic cardiomyocytes that are just initiating beating (32).

## Discussion

Our data demonstrate that a unique combination of transcription factors, ETS2 and MESP1, reprogram fibroblasts into cardiac progenitors, as modeled in Fig. 6. Our model shows that elevated levels of ETS2 drive the conversion of NHDFs into highly replicative cells that express the mesoderm cell-surface marker, KDR. As a critical cardiopoiesis factor (Fig. 1), ETS2 overexpression in NHDF activated the Activin/Nodal pathway but blocked the appearance of smooth-muscle genes, most likely through the inhibition of the BMP-SMAD signaling pathway (Fig. 2). The levels of MESP1 activation by overexpressed ETS2 seemed to be insufficient for reprogramming into cardiac progenitors because the two proteins have to be coexpressed at higher levels to elicit the full reprogramming effect. Core components in the Activin/Nodal program (*ACVR2B*, *TDGF1*, *CITED1/2*, and *LEFTY1*) and core cardiac transcription factors, including

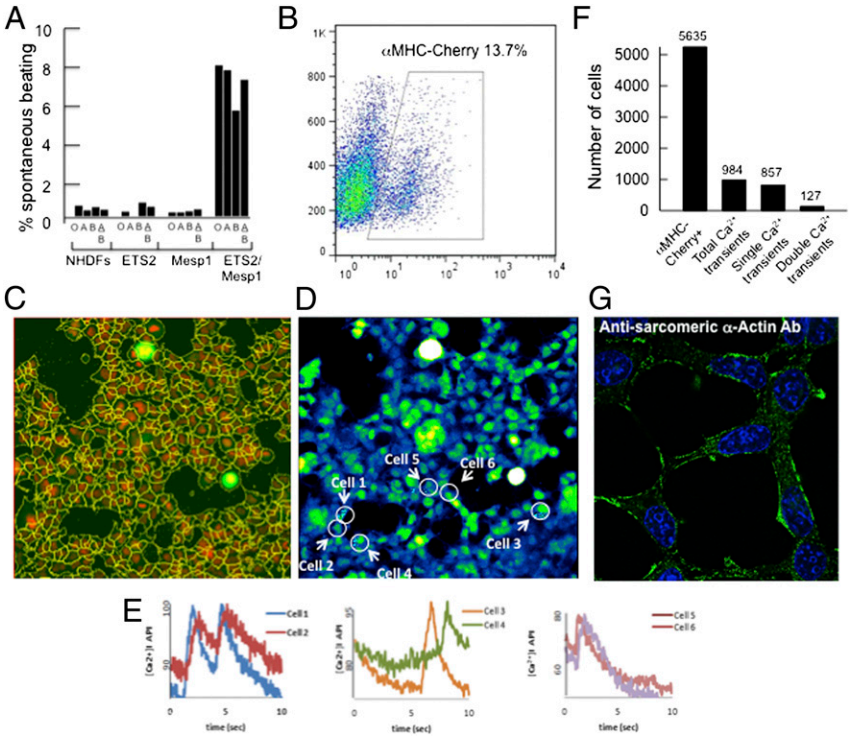


**Fig. 5.** ETS2 and MESP1 converted NHDFs into immature contractile cardiomyocytes. (*A*) Percentages of spontaneously beating cells after Dox-induced conversion of NHDFs by ETS2 and MESP1 in the absence (O) and presence of Activin A (A) and BMP2 (B) were tested by KIC. (*B*) TAT fusion proteins converted about 13.7% of the NHDFs into FACS-sorted α-MHC-Cherry$^+$ cells. Spontaneous beating was observed by KIC imaging in 8–9% of the total cellular population. (*C*) KIC image segmentation for single-cell $Ca^{2+}$ analysis with yellow lines showing cell boundaries. Magnification, 20×. (*D*) Snapshot of the cells during recording their $Ca^{2+}$ transients. Indicated are positions of cells one to six whose $Ca^{2+}$ transients are represented in *E* and whose kinetics of the Fluor-4 fluorescence intensity can be visualized in Movies S1, S2, and S3. Magnification, 20×. (*E*) Single-cell $Ca^{2+}$ traces for the cells identified in *D* recorded in 10-s intervals. (*F*) Characterization of KIC-analyzed cells for total numbers of α-MHC-Cherry$^+$ cells analyzed, total amount of cells in which $Ca^{2+}$ transients were detected, the cells with a single $Ca^{2+}$ peak, amount of cells with two or more $Ca^{2+}$ peaks. (*G*) Many of the α-MHC-Cherry$^+$ cells stained with an α-striated actin antibody displayed periodic staining. Magnification, 40×.

*NKX2.5*, *MESP1/2*, *MEF2C*, *GATA4*, and *TBX20* were also stimulated by ETS2. However, these KDR$^+$ cells did not express smooth muscle actin (*SMA/ACTA2*), *SM22α/TGLN*, or induce terminal cardiogenesis. It remains to be found if ETS2 is a unique cardiogenic factor. Recently, Palencia-Desai et al. (33) showed that the ETS factor, Etv2/Etsrp/ER71, is also a crucial regulator of vascular endothelial differentiation in zebrafish and mouse embryos and inhibits myocardial differentiation. In addition, finding multiple ETS transcripts ETS1, FLI1, ETV1, ETV5, ERG, and ETV6 among the most abundant in the early embryonic heart (23) begs the question for future studies, whether other cardiac-enriched ETS factors could functionally substitute for ETS2.

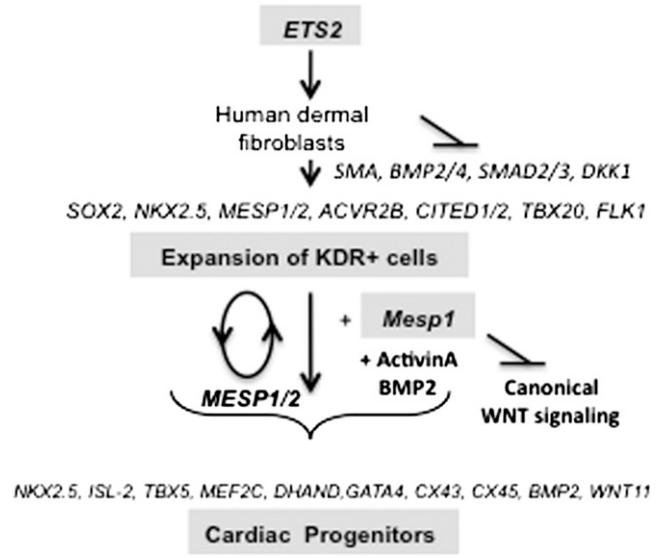


**Fig. 6.** Model for reprogramming of human fibroblasts into cardiac progenitors induced by overexpression of ETS2 and MESP1 or treatment with purified proteins, as supported by data from our study.

MESP1 also activated some of the core cardiac transcription factors (Fig. S3) but failed to convert fibroblasts into cardiac progenitors, unlike its primary responsibility for lineage specification of ES cells (34). Upon coexpression with ETS2, MESP1 switched on the cardiac-specified gene program, in concert with Activin A and BMP2, thereby reprogramming fibroblasts directly into cardiac immature myocytes (Figs. 3 and 4). Considerable numbers of NHDFs were reprogrammed, as manifested by changes in the cell morphology, transcription profile, and cardiac-specific reporters. We observed significant up-regulation of core cardiac factors *NKX2.5*, *ISL1*, *GATA4*, *HAND2*, *MEF2C*, and *TBX20*, mesoderm signaling factors *BMP2*, *TDGF1*, *ACVR2b*, and *CITED1*, as well as contractile factors *MYL2*, *MYH6/α-MHC*, and *MYH7/β-MHC*. In addition, recently forced expression of core cardiac factors TBX5, GATA4, MEF2C, and HAND2 (1, 2) in dividing noncardiomyocytes in mice reprogrammed these cells into functional cardiac-like myocytes, improved cardiac function, and reduced adverse ventricular remodeling following myocardial infarction. It will be important to test whether ETS2 and MESP1, upstream regulators of these core cardiac factors, would also be sufficient to reprogram noncardiomyocytes in the injured heart, because it may reduce the number of factors required for cardiac cell reprogramming.

When the embryonic mouse heart develops from a "primitive heart tube" at E8.5 to a well-defined tubular shape expanded at the ventricular end at E9, $Ca^{2+}$ activity increases from 50% to almost 100% of the nascent myocytes (35) and electrophysiological maturation of human ES-derived cardiomyocytes involves the development of more rapid spontaneous $Ca^{2+}$ transients (36). In comparison, the ETS2/MESP1 reprogrammed cells appear to be at the early to intermediate stages of cardiomyocyte maturation. The ETS2/MESP1-transdifferentiated cells can be considered cardiac progenitors at a baseline that may need to be further maturated into terminal differentiated cardiomyocytes, likely by using cocultures with cardiomyocytes and stimulation with protein factors and small molecules (25, 37). However, as suggested by Song et al. (2), the native environment of the intact heart may be more beneficial than plastic tissue culture dishes for functional reprogramming.

## Materials and Methods

**Cells and Lentiviral Constructs.** Mouse E14 wild-type and *Ets2*$^{-/-}$ mutant ES cells were previously described (38). NHDFs were from Lonza (cc-2509). Human

*ETS2* cDNA (Open Biosystems; clone ID 3852274) and mouse *Mesp1* cDNA (a gift from Y. Saga, National Institute of Genetics, Mishima, Japan) were PCR-amplified with an addition of the HA-tag to the gene ends and cloned into pWPI-GFP and pNL-TREPitt-EGFPΔU3 (pDox-GFP) lentivectors for constitutive and Dox-controlled expression, respectively. The Dox-regulated vector system contains the second vector rtTA2 coding for a reverse tetracycline-dependent transactivator. The α-MHC-Puro and α-MHC-Cherry vectors for gene expression driven from the cardiac specific α-MHC promoter and enhancer were previously described (30). A pWPI-based lentiviral vector NKX2.5-tdTomato has a tandem dimer Tomato red fluorescent protein cDNA (a gift from R. Tsien, University of California, San Diego, CA) and puromycin-resistance gene, both under the control of cardiac-specific composite NKX2.5 enhancer/HSP68 promoter. The pLenti-MLC2v-eGFP-PGK-Hygro reporter vector was obtained by replacing the CMVtight promoter in Addgene plasmid 26585 with a 250-bp fragment of the rat MLC2 promoter (39).

**Reprograming Fibroblasts into Cardiac Progenitors.** Reprogramming of about 80% confluent NHDF into proliferative state was accomplished by infection with pWI-ETS2-GFP and pWPI-Mesp1-GFP vectors for constitutive expression, or with pDox-ETS2-GFP and pDox-Mesp1-GFP for inducible gene expression. To differentiate NHDFs into cardiac progenitors, cells were first infected with NKX2.5-tdTomato reporter and rtTA2 viral vectors, and then infected with pDox-ETS2-GFP and pDox-Mesp1-GFP and cultured in α-MEM with 10% (vol/vol) FBS, 0.1 mM β-mercaptoethanol, 2 mM glutamine, and 0.1 mM non-essential amino acids. Cultures were treated daily with 400 ng/mL Dox for 2, 4, 6 d, then treated with a combination of Activin A (5 ng/mL) and BMP2 (10 ng/mL) for 2 d and cultured in α-MEM with supplements. RNA for qRT-PCR analysis was collected at 8 d after beginning of Dox treatment.

**Protein Transduction of Reprogramming Factors into NHDF with Purified TAT-ETS2 and TAT-MESP1.** Full-length human ETS2 and human MESP1 cDNA were cloned into pTAT-HA vector (28). TAT-ETS2 and TAT-MESP1 proteins were expressed in BL21pLysS *E. coli* cells, purified by 6xHis affinity chromatography on Clontech Talon columns, then desalted into PBS/20% (vol/vol) glycerol buffer using GE PD-10 columns and flash-frozen in dry ice-ethanol mix. Aliquots of the desalted proteins were used to estimate their concentrations from band intensities in SDS/PAGE. Protein solutions were thawed, diluted with culture medium, and sterilized by filtration. NHDFs preinfected with NKX2.5-tdTomato reporter were treated with proteins at 50 nM daily for 4 d and grown in StemPro hES SFM (Invitrogen) over collagen-coated Petri dishes. Further treatment with Activin A/BMP2 and culture maintenance were as described for the Dox reprogramming protocol.

**PCR, Immunofluorescence, and FACS Sorting.** RNA was isolated from cells by standard techniques and subjected to RT- and qRT-PCR, or hybridized against Affymetrix array 430a2 chips for microarray analysis. For immunofluorescence, cells were cultured on glass cover-slips, fixed with paraformaldehyde, and incubated with appropriate antibodies. For FACS analysis, cells were trypsinized after 8 d of transdifferentiation and sorted using an Aria SORP (Becton Dickinson). Antibodies for Western blot, immunofluorescence, and FACS were from the following sources: anti-HA tag (sc-805; Santa Cruz), anti-α sarcomeric actin (ab28052; Abcam), and anticardiac troponin T (ab8295; Abcam), antitroponin I (#4002; Cell Signaling), anti-MHC (MF20; DSHB, University of Iowa), anti–GAPDH-HRP (sc-20357 HRP; Santa Cruz), secondary antibody anti-Rabbit IgG-HRP (sc-2301; Santa Cruz), anti-CD31 (560984; BD Pharmingen), and anti-CD309 (560872; BD Pharmingen).

**KIC Analysis.** The α-MHC sorted cells after transdifferentiation were imaged with a Vala Science KIC instrument that robotically inserts electrodes and registers fluorescent images for individual spontaneously or electrically stimulated cells in α-MHC-Cherry, Hoechst 33342 (nuclei), and Fluo-4 (intracellular $Ca^{2+}$) channels.

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